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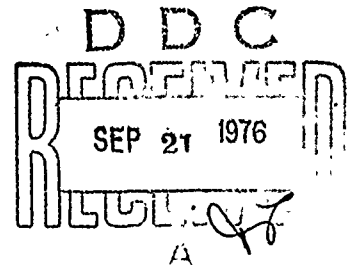
Studies on Macaca mulatta Infected with Rocky Mountain Spotted Fever

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Running title: RMSF in the Rhesus Monkey



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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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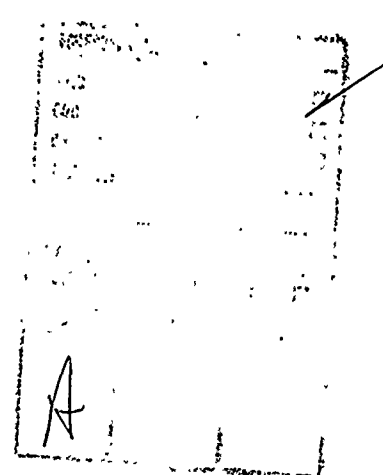
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cortisol, α_1 -acid glycoprotein and haptoglobin increased, whereas albumin decreased during the disease. No significant changes were observed in arterial pO_2 . Cholesterol remained unchanged. The increase in arterial pH and decrease in pCO_2 indicated that respiratory alkalosis was present in monkeys acutely infected with Rickettsia rickettsii. 



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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

SUMMARY

Acid-base alterations and changes in other selected serum constituents (free fatty acids, triglycerides, cholesterol, copper, cortisol, α_1 -acid glycoprotein, haptoglobin and albumin) were measured during a study in 16 male rhesus monkeys to determine the median lethal dose (LD_{50}) of Rocky Mountain spotted fever (RMSF) rickettsiae. The LD_{50} in monkeys of the yolk-sac-grown seed stock was $10^{1.35}$ plaque-forming units. Blood samples were taken from unanesthetized monkeys conditioned to repeated handling. Arterial pH increased and pCO_2 decreased in monkeys during the febrile period. Free fatty acids, triglycerides, copper, cortisol, α_1 -acid glycoprotein and haptoglobin increased, whereas albumin decreased during the disease. No significant changes were observed in arterial pO_2 . Cholesterol remained unchanged. The increase in arterial pH and decrease in pCO_2 indicated that respiratory alkalosis was present in monkeys acutely infected with Rickettsia rickettsii.

A subhuman primate model for studying the pathophysiology of Rocky Mountain spotted fever (RMSF), a rickettsial disease, has been developed using Macaca mulatta.²⁸ Commonly measured blood serum constituents, hemogram and pathologic changes associated with RMSF in rhesus monkeys have previously been reported.^{20,28} However these earlier studies did not include effects of RMSF on acid-base balance. During the febrile period of bacterial sepsis in man, respiratory, metabolic, or mixed alkalosis has been noted.¹³ Moreover, serial sampling of rats during pneumococcal sepsis demonstrated respiratory alkalosis which was attributed to fever and induced hyperventilation.¹⁰ Since RMSF in man is generally studied only in scattered individual cases, it is appropriate to use the rhesus monkey model for additional prospective investigations to learn more about the disease. In order to identify additional alterations of physiologic and metabolic functions attributable to RMSF, the present study was initiated with a multifold purpose. The first was the examination of serial changes in blood acid-base balance throughout the course of RMSF in the monkey. The second was to measure selected serum chemistry variables known to change during generalized infectious diseases in man. It was anticipated that this study would contribute to the basic understanding of this disease process and establish areas of similarity or differences between RMSF and other more commonly studied severe infections. It proved possible to obtain this information during a study in monkeys designed primarily to establish a median lethal dose (LD_{50}), a value required for testing the effectiveness of new RMSF vaccines in monkeys.¹⁶

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Materials and Methods

Rickettsiae--The Sheila Smith strain of Rickettsia rickettsii was propagated in the yolk sac of 5-day-old embryonated chicken eggs. Seed stock was stored at -70 C as infected yolk sac diluted 1:2 in sucrose-phosphate-glutamate (SPG) buffer, pH 7.2. Serial tenfold dilutions of viable rickettsial seed stock were made in SPG buffer containing 2% human serum albumin and titrated by plaque assay on chick fibroblasts, according to the method of Weinberg et al.³² One milliliter, which contained 10^1 , 10^2 , or 10^4 plaque-forming units (PFU)/ml of yolk-sac-grown R rickettsii, was inoculated subcutaneously into each monkey. Rickettsemias were confirmed by the plaque assay method as described by Wike and Burgdorfer.³³

Laboratory Animals--Sixteen well-conditioned, healthy male M mulatta weighing 3 to 4 kg were used. Monkeys were housed in individual cages and fed a commercial ration and water ad libitum. Between 8 and 9 a.m., prior to feeding, rectal temperatures were recorded and blood samples were obtained from the femoral artery for blood gas analysis, and from the femoral vein for hematology and serum chemistry. Anesthesia was not used, since the monkeys had been conditioned to accept daily handling. Blood samples obtained for blood gas analysis were drawn carefully into heparinized syringes. Base-line rectal temperatures, blood gases, hematologic, and serum chemistry values were established during the 2 week period prior to inoculation of the monkeys. For 3 weeks after inoculation of rickettsiae, rectal temperatures were recorded daily and blood samples obtained according to a predetermined schedule for blood gas analysis and for hematologic and rickettsemia determinations. One additional uninoculated monkey served as the control for the blood

gas analyses. Monkeys were arbitrarily considered febrile when their rectal temperature ≥ 40 C.

Analytic Techniques--Blood pH, pCO_2 , and pO_2 were determined at 37 C on a Corning 160 pH/blood gas analyzer. All individual values were corrected for increased body temperature.^{9,26} Rectal temperatures and pH, pCO_2 , and pO_2 values were averaged each day data were available. Mean values for febrile monkeys were compared to their mean base-line control data by one-way analysis of variance and least significant difference. Differences were considered significant when $P < 0.01$. LD_{50} was determined by probit analysis.

The hemogram included total and differential leukocyte counts and packed cell volume. Serum from clotted blood was stored at -70 C. Copper (Cu) analysis was done by flame spectrophotometry.^a Total protein (TP) was determined using refractometry.^b Specific serum proteins were analyzed using an automated immunoprecipitin system utilizing available human serum standards and human antiserum.⁶ Thus, the monkey serum α_1 -acid glycoprotein, haptoglobin, and albumin values are reported as per cent of standard. Triglyceride concentrations were determined using an automated fluorometric system;²⁷ cholesterol and free fatty acids were measured using an automated colorimetric system.⁸ A commercial human radioimmunoassay system was used to assay cortisol.^c

Preinoculation serum values were established for analytical tests. The mean base-line value ± 2 standard deviations (SD), or the range actually observed, was defined as the "limits" for each measured constituent.

^aPerkins-Elmer, Mountain View, California 94040.

^bAmerican Optical, Atlanta, Georgia.

^cSchwartz/Mann, Orangeburg, New York 10962.

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Results

Fifteen of the 16 M mulatta inoculated with R rickettsii became febrile and 14 had detectable rickettsemia (Table 1). The LD₅₀ of yolk-sac-grown seed stock was determined to be $10^{1.35}$ plaque-forming units. Despite differences in dose, mean incubation period, duration of fever and days to death were similar to previous reports.^{20,28} Clinical signs of RMSF included anorexia, lethargy and weakness. During the course of the study, 12 monkeys developed a characteristic rash 3 to 5 days after onset of fever and died 8 to 12 days after inoculation.

Data from one inoculated, but nonfebrile, monkey and the uninoculated control monkey were within the preinfection mean \pm 2 SD. All monkeys were conditioned to repeated handling, hence hyperventilation was not observed during the control periods. As shown in Table 2, rectal temperatures of febrile animals were significantly ($P < 0.01$) increased on days 5 through 9. Arterial pH was significantly ($P < 0.01$) increased over base-line values on day 6 and then remained elevated above the base-line level through day 9. Arterial pCO₂ was slightly lower on days 5 through 9, but was significantly higher on day 3. Alterations persisted if death occurred. There were no significant changes in arterial pO₂. All blood acid-base values of the 3 surviving monkeys returned to base-line values by day 11 of infection and remained there through the end of the study.

Febrile monkeys, particularly those that died, developed slight leukocytosis accompanied by a marked lymphopenia and neutrophilia similar to previous RMSF studies in this laboratory.²⁸ Packed cell volumes of the febrile monkeys were also decreased during the febrile period. Serum free fatty acids, triglycerides, cortisol, copper, α_1 -acid glycoprotein and haptoglobin were increased above normal limits in 73 to 100% of the

febrile monkeys (Table 3) and with the exception of cortisol, remained elevated until death. Albumin and total protein decreased in 87 and 47% of the febrile monkeys respectively and this decrease persisted until death. Cholesterol did not change. Elevated cortisol values in 6 monkeys returned to their individual base-line values 3 to 5 days before death. Altered serum constituents in the 3 surviving monkeys returned to normal limits within 15 days postinoculation.

Necropsies performed on all monkeys that died demonstrated gross lesions typical of RMSF.²⁰ There was widely disseminated, macular, dermal rash characterized microscopically by inflammation of vessels and occasionally accompanied by thrombosis. Other microscopic lesions included minimal interstitial pneumonitis in 7 of 12 monkeys and minimal inflammatory lesions of the adrenal glands in 9 of 12 monkeys.

Discussion

The basic clinical and hematologic responses of the M mulatta to R rickettsii infection were similar to those reported previously.²⁸ Increased arterial pH and decreased pCO_2 indicated the presence of respiratory alkalosis in monkeys with RMSF. The monkeys had been successfully conditioned to handling, for at no time did nonfebrile monkeys demonstrate hyperventilation. The respiratory alkalosis was probably due to fever and its induced hyperventilation, as the onset of acid-base changes was coincident with fever. Base-line arterial pH values in this study were compatible with previously reported values;⁷ we believe that the fever-related trend toward increased arterial pH is a valid finding in this study. Respiratory alkalosis has also been reported during induced systemic pneumococcal infection of rats,¹⁰ and in acute Falciparum malaria in man.³⁰ This fulminant RMSF infection is not accompanied by pulmonary lesions which might interfere with exchange of gases.²⁰ On the other hand, monkeys infected with RMSF were febrile for 3 to 8 days and 58% of the monkeys which died had minimal interstitial pneumonitis upon necropsy. With the increased length of illness there was more time for development of pathologic changes in the lungs which could interfere physically with the exchange of gases. However, respiratory alkalosis developed early during the disease and persisted until death despite the length of illness, pulmonary lesions, and metabolic changes which would favor the development of metabolic acidosis. Two reports concerning RMSF in man describe bronchopneumonia with a secondary bacterial infection.^{15,19} The observed increase in arterial pCO_2 on day 3 of infection in these monkeys cannot be explained or related to other pathophysiologic events.

It has been demonstrated that plasma lipids are elevated in patients with various infectious diseases. The specific patterns of response appear to be related to the causative microorganism and the severity and duration of infection.³ Monkeys with RMSF developed elevated free fatty acids and triglycerides while maintaining normal concentrations of cholesterol. Increased triglyceride levels have been found in rhesus monkeys with malaria,¹ Streptococcus pneumoniae, and Salmonella typhimurium sepsis,¹¹ and in man during gram-negative bacterial infections.¹² Variable levels of free fatty acids have been described in rhesus monkeys during S pneumoniae and S typhimurium infections,¹¹ while increased levels of free fatty acids have been described during gram-negative sepsis in man.¹² Although cholesterol values are depressed in monkeys during both pneumococcal and Salmonella infections,¹¹ serum cholesterol apparently was not altered during RMSF in rhesus monkeys. Increases in free fatty acids and triglycerides may be representative of an increased fatty acid mobilization from adipose tissue and subsequent increases in rates of triglyceride synthesis, or release, or both, by the liver.¹¹ During RMSF, febrile monkeys are partially to totally anorectic and thus have a demand for energy which can be supplied by utilization of free fatty acids from peripheral tissues.

In monkeys, serum cortisol generally increased in response to acute RMSF.² The multifocal, minimal adrenalitis present in 75% of RMSF infected monkeys which were necropsied was similar to lesions reported previously.²⁰ Harrell reported lesions in the adrenal gland of RMSF patients but concluded circulatory collapse arose from peripheral vascular damage rather than adrenocortical failure.¹⁵ Adrenal lesions in monkeys infected with RMSF were apparently neither severe enough to

alter the characteristic glucocorticoid response to infection nor to alter the course of the disease.

Almost all of the copper in serum is tightly bound to ceruloplasmin, a protein synthesized within the liver¹⁸ and also found to be increased during generalized infections.⁴ Increased levels of serum copper seen in this study agree with increased plasma levels generally associated with bacterial and viral infections in man and experimental animals.^{23,25,29,31}

Alterations in the plasma acute-phase reactant protein patterns have been identified during the course of various acute microbial infections.^{5,14,17,21,22,24} Haptoglobin and α_1 -acid glycoprotein are 2 members of the acute phase group which have been found to increase with the onset of most febrile infections in man and to remain increased during the febrile phase of typhoid fever.⁶ In contrast, plasma albumin concentrations have consistently been found to decrease during various acute infectious diseases.⁶ While plasma albumin decreased in the monkeys with RMSF, the vascular lesions associated with this disease could have contributed to the observed decrease.¹⁵ It is probable that the alterations of albumin during RMSF are attributable both to the presence of an acute infectious disease and the complicating vasculitis particularly characteristic to RMSF.

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TABLE 1--Clinical response of 16 M mulatta to R rickettsii infection

Clinical response	No. of responders (range)	Mean number of days (range)
Incubation	15	4.7 (3-9)
Rickettsemia	14	ND*
Fever	15	5.5 (3-8)
Rash	12	(3-5)**
Death	12	(8-12)

*ND = not done.

**Onset.

TABLE 2--Mean rectal temperatures and arterial acid-base alterations in rhesus monkeys which developed fever during R rickettsii infection

Day	No. of monkeys	Rectal temperature °C	pH	pO ₂ (mm Hg)	pCO ₂ (mm Hg)
-4	15	39.3	7.33	100.0	24.4
-1	15	39.2	7.34	102.5	25.1
1	15	39.2	7.36	102.4	26.5
3	15	39.3	7.38	96.0	33.9*
5	15	40.8*	7.38	105.3	22.8
6	15	40.6*	7.46*	92.0	24.0
7	15	40.6*	7.50*	103.2	20.3
8	12	40.3*	7.52*	103.0	21.7
9	10	40.4*	7.47*	95.3	22.0
10	6	39.4	7.32	109.6	30.8
11	4	39.3	--	--	--
12	4	39.2	--	--	--
13	3	--	7.45	81.6	30.0
15	3	--	7.33	99.2	--

*P = <0.01 compared to preinfection days -4 and/or -1, by Analysis of Variance.

TABLE 3--Effect of R rickettsii infection on serum components of febrile rhesus monkeys

Component	% Showing change	Mean preinfection value (+ SD)	Limits measured in normal monkeys
<u>Increase</u>			
Free fatty acids	80	209.7 (92.7) mEq/l	24-398 mEq/l
Triglycerides	93	34.8 (14.0) mg/dl	7-63 mg/dl
Cortisol	73	9.3 (5.3) µg/dl	2-20 µg/dl
Copper	93	120.8 (19.1) µg/dl	82-159 µg/dl
α ₁ -acid glycoprotein	100	<10% of standard*	<10%
Haptoglobin	80	23.0% (9.4%) of standard	<10-42%
<u>Decrease</u>			
Albumin	87	38.4% (4.3%) of standard	30-47%
<u>No change</u>			
Cholesterol	0	164 (28.1) mg/dl	108-220 mg/dl

*Results expressed as percent of human standard used in test.